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(54) Title: METHODS AND MATERIALS FOR GENERATING SH3 DOMAINS WITH TAILORED BINDING PROPERTIES

(57) Abstract: The present invention relates to controlled modification of Src homology region 3 (SH3) domains by using random manipulation of the non-conserved region of SH3 RT-loop. Consequently, the invention concerns methods and materials for generating SH3 domains with engineered binding properties, and their use as tools in research, diagnostics, therapy and drug discovery.

Methods and materials for generating SH3 domains with tailored binding properties

Field of the invention

5

A number of biological processes that are important for normal and pathological states are governed by interactions of cellular proteins mediated by Src homology region 3 (SH3) domains. This disclosure concerns methods and materials for generating SH3 domains with engineered binding properties, and their use as tools in research, therapy, 10 diagnostics and drug discovery.

Background of the invention

Virtually all aspects of cellular behavior, such as adaptation of a cell in response to 15 extracellular stimuli by changing of its pattern of gene expression, are regulated and executed by dynamic and ordered proximity of cellular proteins. During the evolution several different types of protein domains specialized in mediating such regulated and specific protein-protein interaction events have emerged. Protein domains of one type typically form large families of homologous but sufficiently divergent members, such 20 that each of them have unique, although often overlapping specificities for ligand binding.

The SH3 domain was first identified as a region of homology among the Src family tyrosine kinases encoded by oncogenic retroviruses and their cellular proto-oncogene 25 counterparts. Thereafter SH3 domains have been noticed in a large number (>50) of proteins that serve important functions in regulating cell growth, differentiation, and other processes. Because of these functions SH3 domains are intimately involved in pathogenesis of various diseases, in particular cancer. In addition, various microbial pathogens, such as HIV, exploit SH3-mediated processes as a part of their life-cycle. 30 Ability to influence protein complex formation mediated by SH3 domains would therefore have significant therapeutic potential.

SH3 domains are globular protein modules typically consisting of 50 - 70 amino acids found in many different proteins, particularly proteins involved in cellular signal transduction (Cohen et al. 1995. Cell, 80, 237-248; Dalgarno et al. 1997. Biopolymers, 43, 383-400). SH3 domains mediate inter- and intramolecular interactions by binding to
5   ligands that contain a region with a secondary structure known as the polyproline type II (PPII) helix. These ligands can bind to SH3 domains in two opposite orientations, and typically show the "PxxP motif" consensus sequences RXØPXXP and PXØPXR (Ø is a hydrophobic amino acid, X is any amino acid) (Feng et al. 1994. Science, 266, 1241-7; Lim et al. 1994. Nature, 372, 375-9). The positioning of the conserved basic residue
10   (usually an arginine; R) in the PxxP motif determines in which orientation the ligand binds its cognate SH3 domain. In addition, there are atypical SH3-ligands with PPII helices that do not conform to such consensus rules. A notable example is the PPII region in Src, which is involved in catalytic autoinhibition by binding to the SH3 domain of Src itself, but contains only one of the two prolines that ordinarily define a PxxP-motif (Xu
15   et al. 1997. Nature, 385, 595-602).

Sequence variation in the PPII helix region, involving the consensus as well as the adjacent, non-consensus positions, has been shown to influence the specificity in SH3/ligand complex formation. Examples of preference for targets with atypical PxxP
20   consensus motifs have been provided by studies addressing Abl SH3 ligand selection (Feng et al. 1994. Science, 266, 1241-7; Weng et al. 1995. Mol Cell Biol, 15, 5627-34; Yu et al. 1994. Cell, 76, 933-945), and the CrkN-SH3/C3G peptide complex (Knudsen et al. 1995. EMBO J, 14, 2191-8; Wu et al. 1995. Structure, 3, 215-226). The effect of sequence variation involving the non-consensus residues in the PPII region of SH3-
25   ligands has been best demonstrated by experiments in which distinctive target sequences have been selected for different SH3 domains from libraries of chemically synthesized or phage-displayed random peptides (Sparks et al. 1994. J Biol Chem, 269, 23853-6; Viguera et al. 1994. Biochemistry, 33, 10925-33; Yu et al. 1994. Cell, 76, 933-45). However, despite the above-discussed evidence for specificity, the maximal SH3-binding
30   affinities of short PPII ligand peptides are low, and the relative differences in their binding to different SH3 domains are modest.

By contrast, there is increasing evidence that molecular contacts outside the PPII helix interface can provide significant specificity and strength to SH3-binding. Use of phage-display libraries of longer peptides containing a PxxP motif embedded within random sequence has demonstrated that the flanking residues can increase the selectivity of such

5   ligands, which may show up to 20-to-30 fold differences in their affinities towards different SH3 domains (Rickles et al. 1994. EMBO J, 13, 5598-604; Rickles et al. 1995. Proc Natl Acad Sci U S A, 92, 10909-13; Sparks et al. 1996. Proc Natl Acad Sci U S A, 93, 1540-4). Structural analysis of the interactions of Src-SH3 with two such dodecapeptides revealed that the relatively high specificity and affinity (K_D values 0.54

10   μM and 1.2 μM) of these interactions involved contacts between the flanking residues in the peptides and two loop-like structures in the Src-SH3 domain, which represent regions of high sequence diversity among different SH3 domains and are known as the n-src- and RT-loops (Feng et al. 1995. Proc Natl Acad Sci U S A, 92, 12408-15). Similarly, the specific binding of a rationally designed proline-rich ligand to Abl SH3 (K_D 0.4 μM for

15   Abl vs. 273 μM for Fyn-SH3) could be explained by corresponding molecular contacts with Abl SH3 (Pisabarro and Serrano. 1996. Biochemistry, 35, 10634-40; Pisabarro et al. 1998. J Mol Biol, 281, 513-521).

Another interaction that has been informative in elucidating the basis of SH3 binding

20   specificity, which also emphasizes the role of the RT-loop, is the complex between HIV-1 Nef and the SH3 domain of the tyrosine kinase Hck. Nef is a 27 - 34 kD myristoylated protein of primate lentiviruses (HIV-1, -2, and SIVs), and important for development of high viremia and immunodeficiency in the infected host (Harris. 1996. J Gen Virol, 77, 2379-92; Saksela. 1997. Front Biosci, 2, 606-618). Interestingly, Nef has remarkably

25   selective SH3-binding characteristics. It can bind tightly to the Hck-SH3, showing affinity values of approximately K_D 0.2 μM as measured by surface plasmon resonance (Lee et al. 1995. EMBO J, 14, 5006-15). In contrast to the strong binding to Hck, Nef has almost a 100-fold lower affinity towards the highly homologous SH3 domain of Fyn. Biochemical and structural studies have revealed that the basis of this selectivity lies in

30   the efficient strategy of Nef for recognition of the non-conserved SH3 residues distinctive to Hck, in particular the side chain of an isoleucine located in the RT-loop of Hck-SH3 (Lee et al. 1996. Cell, 85, 931-942). The region that accommodates the Hck-SH3 RT-

loop is composed of multiple non-contiguous parts of the Nef polypeptide, and is located distally from the PPII region in the three-dimensional structure of Nef.

Previous attempts to generate molecules that could compete with naturally occurring SH3-interactions have focused on design or selection from random libraries of peptides and peptide-like molecules that could compete with PPII ligands for their binding to their cognate SH3 domains. Success in such approaches has been reported by a number of groups (see references above). Patent applications for different modifications of this approach have been filed (such as WO 95/24419 and WO 96/03649). However, the relative similarity of the SH3/PPII interface of different SH3/ligand pairs presents a problem for developing highly specific inhibitory molecules. To overcome this problem we have chosen a different approach, which is based on the apparent role of the SH3 domain RT-loop in ligand selection that has been indicated by a number of studies, in particular our previous work on the complex between the HIV Nef protein and the SH3 domain of the cellular Hck tyrosine kinase.

The above observations suggest a general model where regions in SH3 ligands outside the PPII helix region provide specificity and affinity for binding by contacting regions that are divergent among SH3 domains, in particular residues in the RT-loop. Prompted by this concept, in the present invention we have constructed a large library (>130 millions) of Hck-derived artificial SH3 domains, in which six non-conserved, Hck-specific residues in the RT-loop have been replaced by a random hexapeptide (termed RRT-SH3 for randomized RT-loop), and expressed these on the surface M13 bacteriophages in order to identify novel SH3 domains with engineered binding characteristics. We show that phage-display is well suited for presentation and selection of modified SH3 domains, and provide strong experimental support for a role of the SH3 RT-loop as a versatile specificity and affinity determinant.

Consequently, as explained hereinbelow, we have found that by randomly manipulating the amino acid sequence comprising the variable region of the RT-loop (in this case six amino acids of the Hck SH3 domain) it is possible to create artificial SH3 domains that

bind with unnaturally high affinities and with predetermined binding specificities to different ligand proteins.

Summary of the Invention

5   The well-characterized interaction between HIV-1 Nef and the SH3 domain of Hck is one of the tightest known SH3-mediated interactions. We have previously shown that a similar capacity for binding to Nef can be transferred to Fyn-SH3 by engineering Hck-like amino acid substitutions into its RT-loop. The present invention is in the finding that,

10   instead of mimicking the structure of a naturally occurring, known cognate SH3 domain, one can generate SH3 domains with desired ligand binding properties by using random manipulation of the RT-loop sequence combined with a powerful affinity or functional selection. Notably the method described in this invention can be used to identify SH3 domains with unnaturally high affinities specific for proteins known to bind to any

15   naturally occurring SH3 domains, as well as to target proteins that are believed to be SH3 ligands but lack an identified SH3 domain-containing cellular partner.

Consequently, the present invention provides a method for generating SH3 domains with tailored binding properties, artificial SH3 domains (termed RRT-SH3 domains) obtained

20   by such a method for use as efficient tools in research, diagnostics, therapy and drug discovery.

Brief description of the drawings

25   Figure 1. A simplified cartoon depicting the strategy of current invention for creating SH3 domains with altered binding properties. Interactions of SH3 domains, such as Hck-SH3, with their cognate ligand proteins involve a set of molecular contacts between the SH3 domain and a proline repeat (PxxP) motif region in the ligand protein. Although necessary for binding, these PxxP-directed interactions are weak and relatively similar

30   in most SH3/ligand complexes, and therefore provide little selectivity and strength for these interactions.

Figure 2A. and Figure 2B. Data from two representative experiments measuring binding of four different RRT-SH3 domains to immobilized Nef based on their ability to compete with labeled Hck-SH3 (Fig. 2A) or RTT-A1 (Fig. 2B). The assay conditions were essentially the same in both cases (see Methods in Example 1), exept that ten times more

5   Hck-SH3 (66 nM, 2A) than RTT.A1 (7 nM, 2B) was used as a probe. The degree of inhibition of probe binding to Nef caused by the added competitors is shown on the y-axis, and the 50% level is indicated by the horizontal dashed line. The concentration of the competitor relative to the probe (ranging 1:128 to 4:1 for Hck-SH3, and 1:8 to 256:1 for RRT.A1) is shown on the x-axis, and the point where the probe and the competitor

10 were present in equimolar amounts is indicated by the vertical dashed line. As expected, in both cases when the same SH3 domain was used as a probe and a competitor, the inhibition curves passed very close to the point where the dashed lines crossed. All RRT-SH3 domains were tested at least three times, and the summary of these data is shown in Table I.

15

Figure 3. Binding of selected RRT-SH3 domains to a Nef variant carrying a F90R mutation. NefR90 was used a ligand for biotinylated RRT.A1 (66 nM) in the presence of unlabeled SH3 domains at relative concentrations ranging from 1:256 - 16:1. Summary of similar data on all tested RRT-SH3 domains is presented in Table I.

20

Detailed description of the invention

In the prototypic version of the invention presented here we have used polymerase chain reaction (PCR) assisted mutagenesis to produce a large collection of SH3 domains

25 (termed RRT-SH3) that shared their overall structure with Hck-SH3, but contained a random combination of amino acids in place of the six RT-loop residues that constitute a variable region among the otherwise homologous SH3 domains. We generated ≈130 million individual recombinant phagemid-expressed RRT-SH3 domains, which exceeds the theoretical number of all possible combinations of six residue peptides made of the

30 20 different amino acids (20^6 = 64 million). We discovered that a method known as phage-display is well-suited for expression of functional SH3 domains on the surface of bacteriophage particles, and used this method as a selection tool for identification of the

rare RRT-SH3 domains with desired binding properties from among the millions of non-binding molecules. Although at the moment phage-display appears to be the preferred method for such functional selection of the RRT-SH3 domains, the current invention is not restricted to this approach, and a number of other methods could also be used for this

5   task.

As a proof of principle for the usefulness of this approach we showed that it is possible to identify individual RRT-SH3's which bound to HIV-1 Nef with up to 40-fold higher affinities than the already avidly binding (K_D 250 nM) Hck-SH3 domain that served as

10   the backbone of the artificial RRT-SH3 molecules. Such superior affinity provided these molecules an ability to efficiently compete with the Hck/Nef interaction even when present in low concentrations, thus pointing out to obvious therapeutic applications. Notably, we showed that it is possible to identify RRT-SH3 molecules from this Hck-derived library that bind with very high affinities to SH3 ligand protein that do not have

15   significant affinity for the natural, unmodified Hck-SH3. As an example of such a protein we used a mutant version of the Nef protein (NefR90) whose ability to bind Hck-SH3 has been disturbed by a mutation that specifically prevents the accommodation of the native Hck-SH3 RT-loop. These proof-of-principle studies are described below in Example 1 together with methodological details of this prototypic application of the invention.

20

The present invention provides a generally useful method for targeting of SH3 ligand proteins with artificial SH3 domains. Although our results indicate that very different binding specificities can be generated by manipulation of the SH3 RT-loop of a single (Hck) SH3-domain backbone structure, other SH3 domains can be similarly modified by

25   the described methodology, if necessary in order to create high affinity molecules for all SH3 ligand proteins. Also, the engineering of the binding-properties by manipulation of the RT-loop region could be combined with other random or specific modifications of the SH3 domain to further optimize its binding or other physico-chemical properties, such as protease resistance or solubility.

30

Instead of targeting individual, known SH3 target proteins *in vitro*, RRT-SH3 libraries may also be used to target yet unknown SH3 target proteins that serve important roles

in cellular events of interest, such as proliferation and programmed cell death. In this approach, an RRT-SH3 library is introduced into cultured cells using vectors such as recombinant retroviruses, and a cell with a phenotype of interest is selected from among a large population of cells expressing different RRT-SH3 domains, as is routinely done

5   in a strategy well known as "functional cloning", where libraries of cell-derived cDNAs instead of RRT-SH3 domain constructs have been introduced into cells. Isolation of the RRT-SH3 domain responsible for the altered cellular phenotype can then be isolated, and used for similar applications as the RRT-SH3 domains discovered by *in vitro* affinity selection, as well as employed for identification of its specific target protein, which

10 could be a previously unknown SH3-target protein or an already recognized protein that has previously not known to be involved in functions related to the altered phenotype of interest that was selected for.

Several valuable applications for tailor-made SH3 domains described by the present

15 invention can be envisioned. They could be introduced into and expressed in cells in order to activate, inhibit or otherwise regulate or modify the functions of SH3-ligand proteins and their complexes with other proteins. If the RRT-SH3 domains would be equipped with a protein with an appropriate targeting signal, the subcellular localization of their target protein could also be influenced this way. Further, a diagnostic method

20 could be designed for the detection of infectious organisms, detecting the binding of the RRT-SH3 domains to their targets, such as viral proteins, either in cells or cell-free systems.

While the most apparent uses of such approaches are in basic research on SH3-mediated

25 cellular processes, similar applications in gene therapy are also obvious. The relatively small size of an SH3 domain also makes it possible that the engineered RRT-SH3 proteins could be delivered into target cells by fusing them to membrane-penetrating peptides or other such methods. Besides being used as therapeutic agents themselves, structural analysis of the RRT-SH3/ligand complexes could also help in design of non-

30 peptide compounds targeted against the SH3-binding interfaces of various disease-associated cellular proteins. It would thus be possible to design drug candidates stucturally mimicking the RRT-SH3 domains and sharing similar binding properties.

Finally, the ability of RRT-SH3 to interact tightly to the SH3-binding surface of the ligand proteins, and thereby compete with any compounds that bind to the same region, could also be helpful in identifying molecules derived by non-rational drug discovery screens that are targeted against these functionally important regions. It would thus be possible to guide drug development by using a RRT-SH3 to recognize the molecular region in its traget protein that should be targeted by a drug in order to prevent similar interactions of this protein with naturally occurring SH3-domains.

The six SH3 amino acid residues to be replaced according to the invention by an artificial amino acid sequence to generate RRT-SH3 domains correspond to the residues 69 to 74 (EAIHHE) in the human p59 Hck protein sequence (GenBank PID: 2144421), and form the exposed part of a SH3 structure known as the RT-loop. Although SH3 domains are divergent in their amino acid sequence in this region, analogous residues are present in the RT-loops of all other SH3 domains as well. In other SH3 domains these analogous amino acids can be specified as the residues occupying the six consecutive positions in the polypeptide chain that immediately follow a conserved stretch of amino acids referred to as the ALYDY consensus motif, because this or related sequence can be identified by amino acid alignment in virtually all SH3 domains. For example, in the most amino terminal of the three SH3 domains of the human Nck protein (GenBank PID:88235) these residues are VAQQEQ and occupy the amino acid positions 14 to 19, whereas in the carboxy terminal SH3 domain of the mouse Vav1 protein (GenBank PID: 6755955) these residues are CARDRS and occupy the amino acid positions 794 to 799.

The RRT-SH3 domains obtained can be further modified in their structure, e.g. by shortening the amino acid sequence thereof, without affecting their function, to obtain derivatives of RRT-SH3 domains. Such derivatives may have advantageous properties for expression in cells.

As an example of generating SH3 domains with tailored binding properties we describe below in detail generation of SH3 domains derived from Hck-SH3, being targeted to the HIV-1 Nef protein.

Example 1:   SH3 domains targeted with high affinity to wild-type and Hck-SH3

binding-deficient forms of HIV-1 Nef

Methods

5

Generation of Hck-SH3-containing phagemid vectors

To produce a phagemid for expression of native Hck-SH3, a DNA fragment encoding

a 56 aa Hck polypeptide fragment (NH$_2$-VV...VDSL-COOH) was amplified by PCR

using Pfu polymerase (Stratagene) and primers containing Pst I and Not I sites, and

10   inserted into the corresponding sites in pCANTAB-5EP, a modified version of

pCANTAB-5E (Pharmacia) with a new Pst I cloning site. To create the library of RRT-

SH3 domains, a longer sense PCR primer 5'-AAT CTG CAG GAA TTC GTG GTT

GCC CTG TAT GAT TAT NNN NNK NNS NNK NNK NNS GAC CTC AGC TTC

CAG AAG GGG GAC-3' extending over the RT-loop encoding region of Hck was used

15   (N = C/G/T/A, S = G/C and K = G/T), and the resulting fragment was cloned into

pCANTAB-5EP as described above. A total of 137x10^6 individual recombinant colonies

were obtained by electroporation into *E. coli* TG1 cells, which were pooled and infected

with the M13KO7 helper phage as explained below.

20   **Production of infectious recombinant phages**

Overnight cultures of TG1 cells carrying the phagemid(s) of interest were grown at 30°C

in 2xYT containing 100 μg/ml ampicillin and 2% glucose (2xYT/AG). Cultures were

diluted 1/10 in 2xYT/AG, and incubated with 5 x 10^8 pfu/ml of M13KO7 helper phage

(Pharmacia) for 2 hours at 37°C. The bacteria were pelleted, and the medium changed

25   to 2xYT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (2xYT/AK). After

overnight growth of the double resistant bacteria in this medium at 37°C, the supernatant

containing the recombinant phages was collected, passed through a 0.45 μm filter, and

stored in aliquots at 4 °C until needed.

30   **Phage selection**

Six-well plates were coated with 10 μg/ml GST-Nef (HIV-1 NL4-3 Nef containing a

T71R change to mimic Nef from typical primary HIV-1 isolates; Saksela *et al.*, 1995.

EMBO J, 14, 484-91), GST-NefR90 (see text; Manninen *et al.* 1998. Virology, 250, 273-82), GST-Nef-PA1 (P72A+P75A mutant of Nef; Saksela *et al.* 1995. EMBO J, 14, 484-91) or plain GST in 50 mM sodium carbonate pH 9.6 at 4°C overnight. Nonspecific binding sites were blocked with 5 % milk in PBS/0.05 % Tween 20, and the wells washed

5. briefly with PBS before 10^7-10^{11} pfu of recombinant phages per well were added (higher titers used in the early rounds of selection), followed by incubation for 2 hours at RT. In some experiments involving NefR90-coated wells, the phage solution was supplemented with 10 µg/ml of soluble wild-type Nef. After incubation with the phages the wells were washed six times (5 min) with PBS + 0.05% Tween 20 and three times

10 with PBS.

In most experiments the bound phages were eluted with a small volume of PBS containing an excess of (150 µg/ml) of the same Nef protein immobilized in the well. Alternatively, the TG1 cells to be infected were added directly to the washed wells. In

15 both cases, the bacteria were first grown in 2xYT to log phase from an overnight culture, infected with the affinity-selected phages for 2 hours at 37°C, after which a sample of 1% was removed for determination of the infectious titer of the selected phages by plating on ampicillin plates. These plates also served as indicators for the enrichment of specific clones when compared to plates infected with phages from GST-coated wells

20. processed in parallel. The remaining 99% of the infected bacteria were supplemented with 100 µg/ml ampicillin and 2% glucose, and subjected to a subsequent infection with 5×10^8 pfu/ml of M13KO7 helper phages for 2 hours at 37°C, after which they were pelleted and resuspended into an equal volume of 2xYT/AK. After an overnight incubation the amplified recombinant phage supernatants were collected as described

25 above, and used for the subsequent round of selection/infection. Usually after 8 cycles of selection ≥12 colonies were picked for miniprep preparation of phagemid DNA, and their RRT-SH3 insert sequenced using ABI Prism 310 (Perkin Elmer Applied Biosystems).

30 **Expression of recombinant proteins**

Generation of the pGEX vector for bacterial expression of the GST fusion proteins for Nef and Hck-SH3 has been described previously (Saksela *et al.* 1995. EMBO J, 14, 484-

491; Manninen *et al.* 1998. Virology, 250, 273-282). GST-RRT-SH3 vectors were constructed by PCR amplification of the SH3 fragments from the corresponding phagemids, and insertion between the Eco RI and Sal I sites in pGEX-4T-1 (Pharmacia), and verified by sequencing. Expression and purification of the GST fusion proteins in

5   *E. coli* BL21 were carried out by standard methods as recommended by the supplier of the pGEX vectors and glutathione resin (Pharmacia). After elution of the fusion proteins from the resin they were concentrated and their buffer changed to PBS by ultrafiltration in Centrex UF2 columns (Schleicher & Schuell), followed by concentrations measurement using the BioRad (Lowry) method using BSA as a standard. The

10   concentration and integrity of the proteins were further confirmed in Coomassie blue stained SDS PAGE gels. Biotinylation was done using the EZ-Link Sulfo-NHS-LC-Biotin reagent, as suggested by the manufacturer (Pierce), after which these proteins were subjected to 3 rounds of ultrafiltration to remove any free biotin, followed by concentration measurement as described above.

15

Competitive Nef/SH3 binding assay

Nunc Maxisorb F8 strips were coated with the different GST-Nef proteins (200 ng in 100 μl per well) overnight at 4°C, followed by a 30 min incubation at RT with 1.5% BSA in washing buffer (WB; PBS + 0.05% Tween-20) to saturate non-specific protein

20   binding; and washed twice with WB. The unlabeled SH3 proteins used as competitors were diluted into WB that contained 1.5% BSA and a large molar excess of plain GST (4 μM) to minimize any GST-directed dimerization of the fusion proteins. 50 μl of this solution was mixed with an equal volume of the probe (biotinylated SH3 in WB) and added to the wells resulting in a final probe concentration of 66 nM (Hck-SH3/Nef and

25   RRT.A1/NefR90 assays) or 7 nM (RRT.A1/Nef assay). After a 1 hour incubation at RT the wells were washed three times with WB, and added 100 μl of 1:2000 dilution (in WB) of streptavidin-biotin horseradish peroxidase complex (Amersham Life Sciences) per well. The plates were incubated 45 minutes at RT and washed again three times, after which their peroxidase activity was measured using 1,2-phenylenediamine-

30   dihydrochloride (OPD; 0.6 mg/ml; Fluka AB) as a substrate. The enzymatic reactions were stopped after 10 minutes by adding 50 μl of 2 M sulfuric acid, followed by optical density measurement at 492 nm using a Victor 1420 Multilabel Counter (Wallac).

Results

The avid binding of HIV-1 Nef to the Src homology-3 (SH3) domain of Hck (K_D 250
nM) has been shown to involve an interaction between the RT-loop of Hck-SH3 and
5   residues in Nef outside of its prototypic polyproline type II (PPII) helix-containing SH3-
ligand region. Such distinctive interactions are thought to provide specificity and affinity
for other SH3/ligand protein complexes as well. In this invention we constructed and
successfully displayed on the surface of M13 bacteriophage particles a complex library
of SH3 domains, which are derived from Hck but carry a random hexapeptide
10 substitution in their RT-loops (termed RRT-SH3). By this strategy we identified
individual RRT-SH3 domains that can bind to Nef with affinities higher than K_D 10 nM.
Some of these high-affinity RRT-SH3 domains resembled Hck-SH3 in that they bound
much less well to a Nef variant containing an engineered F90R mutation that interferes
with docking of the native Hck RT-loop. In addition, we could also select RRT-SH3
15 domains with an opposite specificity, which were dependent on the Arg^{90} residue for
strong binding, and had a 100-fold lower affinity for unmodified Nef. These results
emphasize the importance of the RT-loop in SH3 ligand selection, and suggest a general
strategy for creating SH3 domains with desired binding properties.

20 **Generation of a phage-display RRT-SH3 library**
To examine if it would be possible to present functional SH3-domains on the surface of
bacteriophages we constructed a phagemid containing Hck-SH3, and by using a M13KO7
helper virus produced recombinant phages expressing it fused to the pIII coat protein.
When these phages were incubated in six-well plates coated with purified GST-Nef,
25 GST-Nef-PA1 (an SH3 binding deficient P72A;P75A mutant of Nef), or plain GST
protein, the titers of infectious phage-particles resisting washes were found to be 2 to 3
orders of magnitude higher in the GST-Nef coated wells as compared to wells coated
with plain GST or with the GST-Nef-PA1 fusion protein (data not shown). Thus, these
results indicated that functional SH3 domains can be displayed on the surface of M13
30 phage particles, and that such phages can be positively selected using a cognate SH3
ligand as an affinity reagent.

To create SH3 domains with novel ligand binding properties, we used PCR-assisted
mutagenesis to produce a large collection of SH3 domains (termed RRT-SH3) that shared
their overall structure with Hck-SH3, but contained a random combination of amino
acids in place of the six RT-loop residues (EAIHHE) that constitute a variable region

5   among the otherwise homologous SH3 domains. We generated ≈130 million individual
recombinant phagemid clones expressing different RRT-SH3 domains, which exceeds the
theoretical number of all possible combinations of six residue peptides made of 20
different amino acids (20^6 = 64 million). An infectious phage stock displaying this
heterogeneous population of RRT-SH3 proteins was generated, as explained above for

10   the Hck-SH3-expressing phagemid, and used for subsequent affinity selection
experiments.

Selection of RRT-SH3 proteins binding to Nef

To test if the RRT-SH3 library contained novel Nef-binding SH3 proteins, we carried

15   out an extensive selection of phages showing affinity for wild-type HIV-1 Nef. The
selection scheme consisted of multiple (11 in all) independent experiments consisting of
7 or 8 rounds of affinity-selection and helper virus-assisted reamplification of the
recombinant phages. A representative collection of RRT-SH3 clones derived from
different rounds of phage selection were sequenced to follow the loss of heterogeneity

20   in the population and the emergence of putative Nef-binding RRT-SH3 clones. A total
of 278 RRT-SH3 clones selected using Nef were sequenced (Table I and data not
shown). After six rounds of selection, the phage populations were typically dominated
by 1 - 3 different RRT-SH3 clones. In most cases these dominant clones were identical
or similar to the clones derived from other independent experiments, leading us to

25   believe that we had rather exhaustively sampled the library for SH3 domains capable of
high-affinity binding to HIV-1 Nef.

The RT-loop sequences of the RRT-SH3 clones found to be dominant after ≥ 7 rounds
of selection are listed in Table I. They fell into four sequence families characterized by

30   three or more identical residues within each class. In addition, these families also shared
obvious similarities, most notably a serine residue in the second of the six (2/6)
randomized positions present in all of them. One prevalent clone (RRT.C1) contained

the consensus motifs of two different RT-loop sequences families (B and C). Besides the frequent appearance of serine, these RT-loops were remarkably rich in aromatic and proline residues. Although the RT-loop of the natural Nef ligand, Hck-SH3, also contains two aromatic residues (histidines) the selected RT-loop sequence did not bear

5   obvious resemblance to this region in Hck-SH3, or any other naturally occurring SH3 domain in the GenBank database.

Table I.   RT-loop sequences and binding of the corresponding SH3 domains to Nef and NefR90

SH3 domain	RT-loop	Hck-SH3 as the probe Ratio of competitor per probe giving 50% inhibition with Nef as the ligand	RRT.A1 as the probe Inhibition at an equimolar probe:competitor ratio	
			Nef as the ligand	NefR90 as the ligand
Hck-SH3	E A I H H E	1:1	<5 %	<5 %
RRT.A1	V S W S P D	1:35	55 %	52 %
RRT.A2	F S W S D T	1:19	20 %	53 %
RRT.A3	D S W S T S			
RRT.A4	Y S W S D M	1:10	34 %	44 %
RRT.B1	W S P F P S	1:26	41 %	9 %
RRT.B2	D S P F S F	1:22	22 %	26%
RRT.B3	F S P F S F			
RRT.B4	F S P F D W	1:21	41%	37%
RRT.B5	S S P F D W			
RRT.B6	Y S P F S W	1:37	51 %	14 %
RRT.C1	T S P F P W	1:31	39 %	<5 %
RRT.C2	Y S F F P W	1:16	20 %	13 %
RRT.C3	Y S D F P W	1:26	42 %	20 %
RRT.C4	D S W F P W	1:14	10 %	19 %
RRT.D1	S S F Y S S	1:22	45 %	17 %
RRT.m1	Q G F L D Q	1:0.8	<5 %	73 %
RRT.m2	N A F L P S	1:2	<5 %	73 %
RRT.m3	E A W S P L	1:17	22 %	62 %
RRT.m4	E S Y S E W	1:0.8	<5 %	46 %

When the RRT-SH3 phage supernatants from the late rounds of selection, which were dominated by the clones shown in Table I, were subjected to an additional round of affinity selection using wild-type Nef and the SH3-binding-negative Nef-PA1 mutant in
5   parallel, efficient capture of recombinant phages was seen only in the former case, whereas phage recovery from the Nef-PA1-coated wells did not differ from the control wells without Nef (data not shown). Thus, while the RT-loop sequences of the enriched RRT-SH3 phages provided them with a superior affinity for Nef as compared to the rest of the clones in the library, these interactions were critically dependent on an intact
10   PxxP-motif in Nef, as expected for *bona fide* SH3-mediated binding.

RRT-SH3 domains can bind to Nef with high affinity

To study the binding properties of the selected RRT-SH3 domains in more detail, we transferred several members from each of the clone families into a bacterial expression
15   vector to be produced for biochemical studies. To estimate their affinities for Nef we developed a competitive 96-well plate binding assay. In this assay serial dilutions of RRT-SH3 proteins were tested for their ability to compete with a constant amount of biotinylated Hck-SH3 for binding to immobilized Nef, followed by colorimetric quantitation of bound Hck-SH3. The binding affinities of the different RRT-SH3 proteins
20   used as competitors could then be easily calculated based on the well-characterized K_D value (250 nM) of the Hck-SH3/Nef interaction. This assay design was chosen because it should be relatively insensitive to most potential sources of error that might easily affect a non-competitive assay, such as effects caused by the GST part of the SH3 fusion proteins or related to the incubation and washing conditions. The validity of this assay
25   was strongly supported by the excellent correlation of the theoretical and experimental values for a homotypic competition of unlabeled Hck-SH3 with biotinylated Hck-SH3, showing close to 50% inhibition at 1:1 ratio, and expected values over a wide range of concentration ratios (Fig. 2A).

30   As evident from Figure 2A and Table I, all RRT-SH3 proteins from late rounds of selection bound to Nef substantially better than Hck-SH3. Many of these RRT-SH3's were able to compete more than 50% of binding of Hck-SH3 to Nef even when present

in a 20-fold lower concentration, indicating a K_D value below 12 nM. Although such avidly binding RRT-SH3 domains were found in all RT-loop sequence families, the Ser-Pro-Phe tripeptide motif was frequently present in clones that had showed the strongest binding to Nef. The proteins RRT.A1, RRT.B6, and RRT.C1 could consistently inhibit

5   50% or more of the Hck-SH3 binding even at concentration ratios lower than 1:35, corresponding to K_D values as low as 7 nM.

These remarkably high affinities were also supported by experiments in which 96-well plate assay was formatted in the reverse configuration, using a high-affinity RRT-SH3

10   domains (RRT.A1) as the probe and Hck-SH3 or one of the other RRT-SH3 proteins as the competitor (Fig. 2B). The ability of the different RRT-SH3 proteins to compete with RRT.A1 correlated well with their relative capacities in inhibiting Hck-SH3 binding. Notably, Hck-SH3 was a very poor competitor of the RRT.A1/Nef interaction, and was able to cause a 50% inhibition only when added in more than a 100-fold excess of

15   RRT.A1 used as a probe (7 nM vs. >0.85 μM). As when using Hck-SH3 as the probe, the homotypic competition of with an equimolar amount of unlabeled RRT.A1 resulted in an expected 50% inhibition of binding. Thus, this reverse configuration assay confirmed our conclusions regarding the superior binding affinities of the selected RRT-SH3 proteins, and suggested that their affinities could be even higher than estimated

20   using Hck-SH3 as the probe.

RRT-SH3 domains recognize Nef by divergent strategies

As discussed above, an isoleucine residue in the RT-loop of Hck-SH3 has been shown to be important for its binding to Nef. The aliphatic side chain of this Ile residue fits into

25   a hydrophobic pocket formed in part by the Nef residue Phe^{90}. Mutation of this phenylalanine into an arginine (creating a mutant referred to as NefR90 in the following) greatly diminishes the affinity of Hck-SH3 binding (K_D 1.99 μM). While this interaction provides a structural explanation for the affinity of Hck-SH3, other modes of molecular recognition of the Nef SH3-ligand surface could also be envisioned. Therefore, we were

30   interested in examining whether the Nef-selected RRT-SH3 domains also depended on a similar hydrophobic interaction, and would therefore also be affected by the F90R mutation in Nef.

Due to the low affinity of the Hck-SH3/NefR90 interaction it was not possible to develop a quantitative 96-well plate assay based on binding of Hck-SH3 to immobilized NefR90. In contrast, strong binding and an adequate signal-to-noise ratio were observed when biotinylated RRT.A1 protein was tested as a probe. This indicated that the RRT-SH3 domain RRT.A1 did not critically depend on the Phe[90] residue in its binding to Nef, and allowed us to examine the relative affinities of the rest of the RRT-SH3 proteins for NefR90 based on their ability to compete with RRT.A1 (Fig. 3).

As expected, Hck-SH3 was a very inefficient competitor of the RRT.A1/NefR90 interaction (Fig. 3 and Table I), and failed to cause a 50% inhibition of binding even when used in a 16-fold molar excess (66 nM vs. 1.1 μM), which was the highest practical competitor:probe ratio in the NefR90-assay. Based on the K_D value 1.99 μM previously measured for the Hck-SH3/NefR90 complex (Manninen *et al.* 1998. Virology, 250, 273-82), the absolute affinity of RRT.A1 for NefR90 was therefore estimated to be 100 nM or better. When the other Nef-selected RRT-SH3 proteins were tested as competitors for RRT.A1 binding, widely varying affinities for NefR90 were observed (Fig. 3 and Table I). Some RRT-SH3's (such as RRT.A2 and RRT.B4) competed efficiently with RRT.A1 binding, indicating that like RRT.A1 they also were relatively insensitive to the F90R change. In contrast, other RRT-SH3's (such as RRT.B1 and RRT.C1) were almost completely unable to interfere with RRT.A1/NefR90 complex formation even when tested at high concentrations. Thus, some of the high-affinity RRT-SH3 proteins shared with Hck-SH3 a mode of binding to Nef in which the Nef Phe[90] plays a dominant role, whereas others appeared to recognize Nef by dissimilar mechanisms, and bound well also to NefR90.

RRT-SH3 specific for the NefR90 mutant

The above finding suggested that, depending on their RT-loops, the selected SH3 domains could recognize ligands by divergent molecular strategies. Encouraged by this observation we wanted to extend this concept further by engineering SH3 domains with entirely redirected binding specificities. To this end, we decided to develop RRT-SH3 domains that would not bind to wild-type Nef, but instead would show strong affinity for the NefR90 mutant. Thus, binding of such RRT-SH3 domains would be strictly

dependent on interactions mediated by structural determinants involving the mutant Arg[90]

residue, and would bear little resemblance to the natural recognition of Nef by Hck-SH3.

For this purpose Nef-F90R was used as an affinity selection reagent, as described above

for wild-type Nef, except that in some experiments an excess of soluble wild-type Nef

5　　　was also included to enhance selection RRT-SH3 proteins that would primarily recognize

Arg[90]-dependent determinants.

The most frequently observed RT-loop sequences of the NefR90-selected RRT-SH3

domains are shown in the bottom panel of Table I. Although these selection experiments

10　　were carried out almost as extensively (6 independent experiments) as in the case of

wild-type Nef, equally obvious consensus RT-loop sequences were not apparent. As seen

in Table I, however, certain amino acids were favored in some positions, such as the

dipeptide Phe-Leu in the positions 3/6 and 4/6, Glu in 1/6, Ala in 2/6, and Ser in 4/6.

Notably, these NefR90-selected RT-loops did not contain any of the sequence motifs that

15　　characterized the Nef-selected RRT-SH3 domains. Also, with one exception (RRT.m4),

the Ser 2/6 that was invariant in the Nef-selected clones was absent from the NefR90-

selected RRT-SH3 domains.

As shown in Fig. 3 and Table I, all RRT-SH3 proteins selected for NefR90 binding

20　　(RRT.m1 through RRT.m4) could efficiently compete with binding of the clone

RRT.A1 to NefR90, and were estimated to have affinities 8- to 4-fold higher than the

"dual-specific" clone RRT.A1. Remarkably, binding of some of these NefR90-selected

RRT-SH3 domains was critically dependent on the mutant Arg[90] residue, whereas others

appeared to be "dual-specific". An example of the latter was RRT.m3, which besides

25　　being a potent inhibitor of the RRT.A1/NefR90 interaction, also competed well with

RRT.A1 in binding to Nef. In contrast, the clones RRT.m1 and RRT.m4 were virtually

unable to compete with RRT.A1 for binding to Nef (Fig. 3 and Table I). Thus, only a

small fraction of the strong affinity of RRT.m1 and RRT.m4 towards NefR90 was

contributed by structural determinants that did not involve the mutant Arg[90] residue. Due

30　　to this residual (Arg[90]-independent) affinity, however, they could still compete with the

Hck-SH3/Nef interaction. Nevertheless, the fact that these clones bound to NefR90 at

least 100-times better than Hck-SH3 or some of the Nef-selected clones (such as

RRT.C1) clearly indicated that they had been provided with a strikingly novel binding specificity, which targeted them for a high-affinity interaction that depended on an artificially created epitope in their ligand.

Claims

1. A method for generating SH3 domains with tailored binding properties, comprising

    a) producing a collection of SH3 domains containing a randomized RT-loop (RRT-SH3 domains),

    b) generating recombinant libraries expressing said RRT-SH3 domains,

    c) subjecting such libraries to affinity or functional selection steps to identify novel SH3 domains.

2. The method according to claim 1, wherein step a) is effected by replacing amino acid residues in the variable region of the RT-loop by any other amino acid residues.

3. The method according to claim 2, wherein said amino acid residues in the variable region of the RT-loop are the six amino acid residues corresponding to the residues 69 to 74 (EAIHHE) in the human Hck protein sequence.

4. The method according to claim 1, wherein the recombinant libraries are selected from plasmid, phagemid and viral libraries.

5. An artificial SH3 domain, wherein amino acid residues in the variable region of its RT-loop have been replaced with any other amino acid residues.

6. The artificial SH3 domain according to claim 5, wherein said amino acid residues in the variable region of its RT-loop are the six amino acid residues corresponding to the residues 69 to 74 (EAIHHE) in the human Hck protein sequence.

7. An artificial SH3 domain derived from Hck-SH3 and targeted to the HIV-1 Nef protein, wherein the peptide motif EAIHHE in the variable region of its RT-loop has been replaced with a peptide motif selected from XSWSXX, XSPFXX and XSXFPW, wherein X is any amino acid.

8. The artificial SH3 domain according to claim 7, wherein X is selected from V, F, D, M, P, S, T, W and Y.

9. The artificial SH3 domain according to claim 8, wherein the peptide motif is selected
5   from VSWSPD, FSWSDT, DSWSTS, YSWSDM, WSPFPS, DSPFSF, FSPFSF, FSPFDW, SSPFDW, YSPFSW, TSPFPW, YSPFPW, YSDFPW and DSWFPW.

10. An artificial SH3 domain derived from Hck-SH3 and targeted to the HIV-1 Nef protein, wherein the peptide motif EAIHHE in the variable region of its RT-loop has
10   been replaced with a peptide motif selected from SSFYSS, QGFLDQ, NAFLPS, EAWSPL and ESYSEW.

11. Use of an RRT-SH3 domain or a derivative thereof in inhibiting, activating, or otherwise modifying the functions of cellular or pathogen-encoded proteins for research
15   or therapeutic purposes.

12. A diagnostic method for detecting an infectious organism in a sample, comprising detecting the binding of an RRT-SH3 domain or a derivative thereof to proteins of such an infectious organism.
20.

13. Use of an RRT-SH3 domain or a derivative thereof in identifying novel protein targets for drug development.

14. Use of an RRT-SH3 domain or a derivative thereof in identifying suitable molecular
25   surfaces in known SH3 ligand proteins for guiding drug development.

15. Use of structural information provided by an RRT-SH3 domain or a derivative thereof for rational design of drugs.

30   16. Method for identifying novel SH3 target proteins, comprising detecting the ability of proteins of interest to bind to an RRT-SH3 domain functionally selected as described in claim 1, or to a derivative thereof.
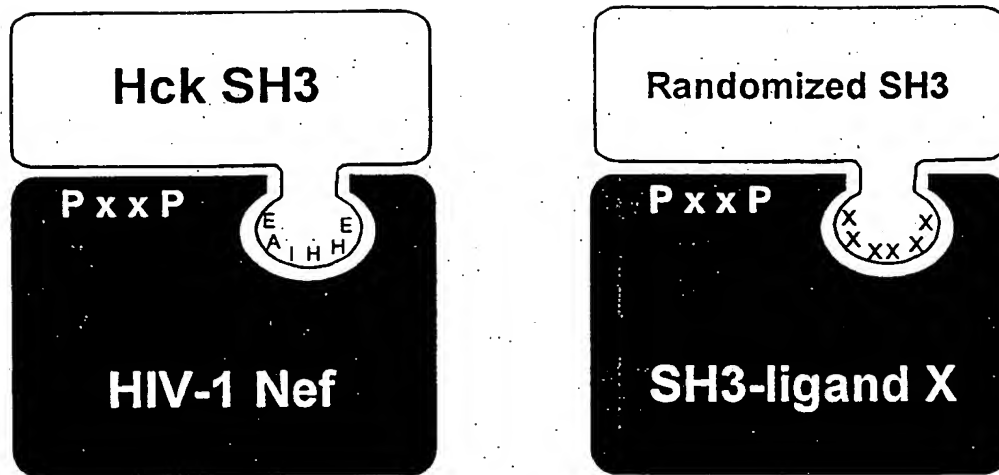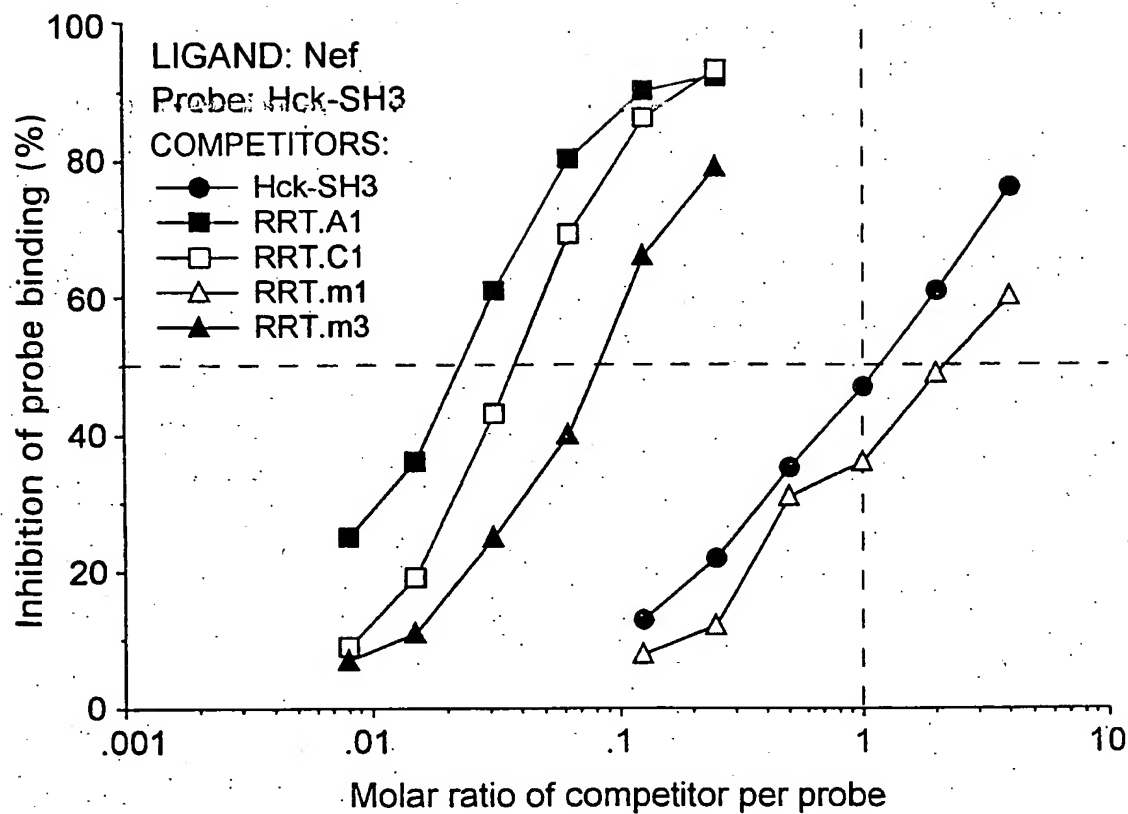
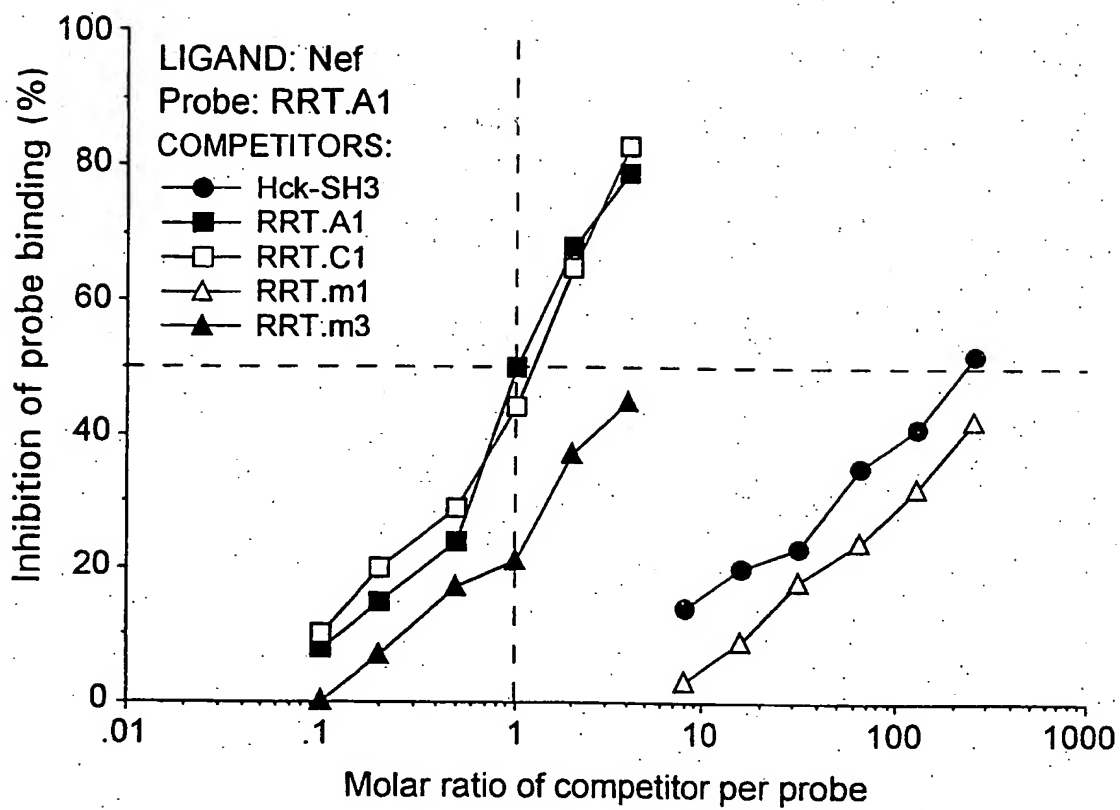Figure 1

Figure 2A

Figure 2B

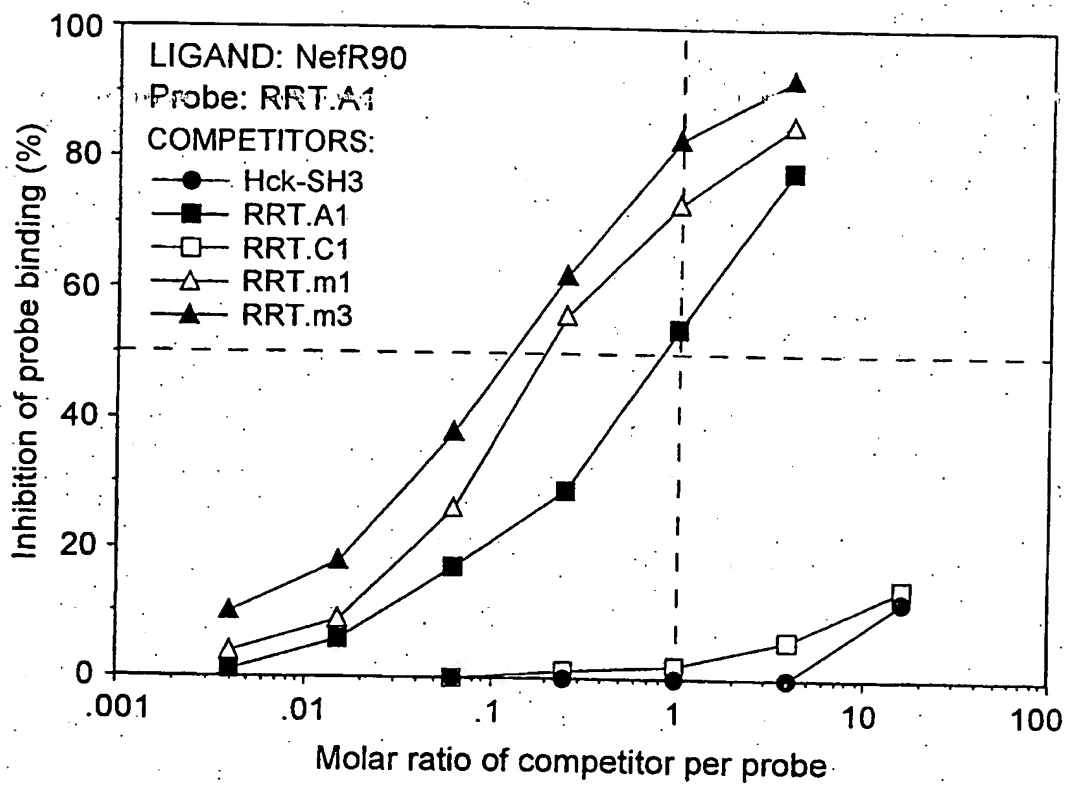Figure 3

SEQUENCE LISTING

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<120> Methods and materials for generating SH3 domains with
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND MATERIALS FOR GENERATING SH3 DOMAINS WITH TAILORED BINDING PROPERTIES

(57) Abstract: The present invention relates to controlled modification of Src homology region 3 (SH3) domains by using random manipulation of the non-conserved region of SH3 RT-loop. Consequently, the invention concerns methods and materials for generating SH3 domains with engineered binding properties, and their use as tools in research, diagnostics, therapy and drug discovery.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/10, G01N 33/53, A61K 38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9631625 A1 (CYTOGEN CORPORATION), 10 October 1996 (10.10.96), see in particular the claims	1-4,11-14,16
A	Biochemistry, Volume 37, 1998, Stefan Arold et al, "RT Loop Flexibility Enhances the Specificity of Src Family SH3 Domains for HIV-1 Nef", page 14683 - page 14691	1-4,11-14,16
A	Structure, Volume 5, October 1997, Stefan Arold et al, "The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling", page 1361 - page 1372, see particular, figure 3	1-4,11-14,16

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 2000	0 1 -12- 2000

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Patrick Andersson/EÖ Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

C (Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages.	Relevant to claim No.
A	EMBO J, Volume 14, No 20, 1995, Lee CH et al, "A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein", page 5006 - page 5015, & Medline accession no. 96067129	1-4,11-14,16
A	EMBO J, Volume 14, No 5, 1995, Erpel T et al, "Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions", page 963 - page 975, & Medline accession no. 95196759	1-4,11-14,16
A	WO 9603649 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL), 8 February 1996 (08.02.96)	1-4,11-19,16
P,X	J Mol Biol, Volume 293, No 5, November 1999, Hiipakka M et al, "SH3 domains with high affinity and engineered ligand specificities targeted to HIV-1 Nef", page 1097 - page 10106, & Medline accession no. 20016380	1-4,11-14,16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI00/00477

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **11-12**
   because they relate to subject matter not required to be searched by this Authority, namely:

   see next sheet

2. ☒ Claims Nos.: **15**
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

   **No specific structural information regarding the RRT-SH region
   is disclosed. Consequently, the claim does not comply with PCT
   art 5(disclosure) or art 6 (clarity).
   The claim has not been searched.**

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July1998)

Box I.1

Claims 11-12 relate to methods of treatment of the human or animal
body by surgery or by therapy/ diagnostic methods practised on the
human or animal body/Rule 39.1.(iv). Nevertheless, a search has
been executed for these claims. The search has been based on the
alleged effects of the compounds/compositions.

Box II

According to PCT rule 13.2, an international application shall
relate to one invention only or a group of inventions linked by one
or more of the same or corresponding "special technical features",
i.e. features that define a contribution which each of the
inventions makes over the prior art.

The claimed invention relates to a method for generating SH3
domains using randomised RT-loops as well as SH3 domains where an
amino acid residue in the RT-loop of a SH3 domain has been
replaced. In Arold S. et al., 1997 see search report, an amino acid
residue in the RT-loop region of Fyn kinase has been replaced, this
residue corresponds to a residue in the human Hck protein residues
69-74. Consequently, there are no unifying special technical
feature between the method claimed and the SH3 domains claimed. The
different Sh3 domains are further divided into different invention
depending on structure.

The following invention were found:
Invention 1, claims 1-4 completely claims 11-16 partially: a method
for generating SH3 domains,
Invention 2, claims 5-9 and 11-16 partially: a SH3 domain where the
RT-loop has been replaced with a peptide motif XSWSXX.
Invention 3, claims 5-9 and 11-16 partially: a SH3 domain where the
RT-loop has been replaced with a peptide motif XSPFXX.
Invention 4, claims 5-9 and 11-16 partially: a SH3 domain where the
RT-loop has been replaced with a peptide motif XSXFPW.
Invention 5(i.e. invention 5-9), claim 10 and claims 11-16
partially: each invention relates to a SH3 domain where the RT-loop
has been replaced with each of the peptide motifs in claim 10
respectively.

Invention 1 has been searched.

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9631625	A1	10/10/96	AU	711141 B	07/10/99
				AU	5382196 A	23/10/96
				CA	2217641 A	10/10/96
				EP	0833941 A	08/04/98
				IL	117840 D	00/00/00
				JP	11509172 T	17/08/99
				ZA	9602813 A	09/10/96
WO	9603649	A1	08/02/96	AU	3146095 A	22/02/96
				CA	2195629 A	08/02/96
				EP	0772773 A	14/05/97
				JP	10503369 T	31/03/98